REMARKS/ARGUMENTS

Upon entry of the instant amendment, claims 1-14X and 24-30 are pending in the instant application. Claims 1, 7-9, 11, 13, 14, 24, 27 and 28 have been amended. Claims 15-23 have been canceled without prejudice. New claim 30 has been added. Applicants respectfully submit that the amendments do not introduce new matter and are made without any intention to abandon the subject matter as filed, but with the intention that claims of the same, greater, or lesser scope may be filed in a continuing application.

Claim Objection

The Examiner objected to claim 11, as being a multiple dependent claim which is dependent from another multiple dependent claim. To clarify claim 11, claim 11 has been amended to depend only from claim 1. Applicant believes that this amendment overcomes the rejections of the Examiner in this regard.

Rejections Under 35 U.S.C. §101

The Examiner rejected claims 17-23 under 35 U.S.C. §101 as the claimed recitation of a use without any process steps results in a claim which is not a proper process claim. Applicant respectfully traverses the rejections; however, in order to expedite the prosecution, Applicant has chosen to cancel claims 17-23 without prejudice. Applicant believes that these cancellations overcome the rejections of the Examiner in this regard.

Rejections Under 35 U.S.C. §112, second paragraph

The Examiner rejected claims 1-29 under 35 U.S.C. §112, second paragraph. Applicant respectfully traverses the rejections.

Claims 1, 13 and 27 were rejected for reciting "derivative thereof". The actual recitation was "quinazolinone derivative". In order to expedite the prosecution, Applicant has chosen to amend claims 1, 13 and 27 to remove the recitation of

"derivative". Applicant believes that these amendments overcome the rejections of the Examiner in this regard.

Claims 1, 13 and 27 were rejected for reciting "the general". The actual recitation was "the general formula I", which was only recited in claims 1 and 13. In order to expedite the prosecution, Applicant has chosen to amend claims 1 and 13 to remove the recitation of the word "general", according to the recommendation of the Examiner. Applicant believes that these amendments overcome the rejections of the Examiner in this regard.

Claims 1, 13 and 27 were rejected for reciting "pharmaceutically acceptable salts". In order to expedite the prosecution, Applicant has chosen to amend claims 1, 13 and 27 according to the recommendation of the examiner to remove the recitation of the plural form of "salts", so as to recite "pharmaceutically acceptable salt". Applicant believes that these amendments overcome the rejections of the Examiner in this regard.

Claims 14 and 28 were rejected for reciting "solvent". In order to expedite the prosecution, Applicant has chosen to amend claims 14 and 28 according to remove the recitation of the word "solvent". Applicant believes that these amendments overcome the rejections of the Examiner in this regard.

The Examiner rejected claims 17-23 for indefiniteness as the claimed recitation of a use without any process steps results in a claim which is not a proper process claim. In order to expedite the prosecution, Applicant has chosen to cancel claims 17-23 without prejudice. Applicant believes that these amendments overcome the rejections of the Examiner in this regard.

Rejections Under 35 U.S.C. § 103(a)

The Examiner rejected claims 1-10 and 12-29 under 35 U.S.C. §103(a) as being anticipated by Pines (WO 98/34613) in view of Nagler (Nagler et al, Am J. Respir. Crit Care, 154, pp 1082-1086, 1996; a copy is attached for the convenience of the Examiner). Applicant respectfully traverses the rejections.

Pines teaches the use of halofuginone alone for treating various malignancies. All of the experimental examples relate to the use of halofuginone alone, without any other treatment, such as chemotherapy or radiation therapy. Pine does not teach or suggest even the possibility of such a combination.

Nagler teaches that halofuginone may be used to treat pulmonary fibrosis after being induced by bleomycin in rats. While bleomycin is an adjuvant chemotherapeutic agent, the rats in the study did not have any malignancy. Instead, bleomycin-induced pulmonary fibrosis was being used as a basic model for pulmonary fibrosis itself; the method of induction of such fibrosis was irrelevant for the study performed. Thus, no effect of bleomycin and halofuginone on cancer was studied, nor was it taught or suggested that such a combination may have a therapeutic effect.

By contrast, the present invention relates to a pharmaceutical composition and a method for treating cancer through the synergistic action of a quinazolinone compound, such as halofuginone, and an anti-cancer therapeutic agent, which may be (for example) a chemotherapeutic agent and/or radiation. Data is shown to support such synergism in the text of the present application; in addition, further data is presented in the attached published article by Sheffer et al (and the present inventors), "Inhibition of fibroblast to myofibroblast transition by halofuginone contributes to the chemotherapy-mediated antitumoral effect", Mol Cancer Ther 2007;6(2). February 2007. This article demonstrates synergistic effects for docetaxel and vincristine with halofuginone for treatment of malignancies, including prostate cancer and Wilms' tumor.

The present invention also relates to a method for alleviating or preventing the damage induced by radiation therapy through administration of a quinazolinone compound, such as halofuginone.

Neither of the above references, alone or in combination, teaches or suggests these aspects of the present invention. As noted above, Pines teaches the effect of halofuginone alone on cancer, so synergism with an anti-cancer agent is not possible; Pines is also silent on preventing or alleviating damage caused by radiation. Nagler teaches that halofuginone may be used to treat the side effects of bleomycin with regard

~~to pulmonary fibrosis; however, this effect, far from being synergistic with bleomycin, is~~ actually working against the effects of bleomycin. Nagler is also silent on damage caused by radiation. There is no motivation to combine these references, as they are not related to treatment of the same or even similar diseases. Furthermore, even if combined, they would fail to teach synergistic treatment of cancer or preventing or alleviating damage caused by radiation.

To more distinctly point out the invention in these embodiments, Applicant has chosen to amend claim 1 to recite that "said quinazolinone compound and said at least one additional anti tumor treatment are effective synergistically". Support for this amendment can be found in paragraphs 1, 21 and 29 of the application as published.

A new dependent claim, claim 30, depending from claim 1, has been added to recite that the "cancer is selected from the group consisting of glioblastoma and pancreatic cancer". Support for this new claim can be found in paragraphs 100-106 and paragraphs 126-127 of the application as published.

Claim 13 has been amended to recite at least one additional anti tumor agent "selected from the group consisting of BCNU, radiation, docetaxel and vincristine, or a combination thereof". These agents were demonstrated to be synergistically effective with halofuginone in experiments published in the above referenced paper or in the present application.

Applicant feels that these amendments and arguments overcome the Examiner's rejections in this regard.

CONCLUSION

Applicant believes that the claims are in condition for allowance. If the Examiner believes that a telephonic interview with the undersigned would expedite prosecution of this application, the Examiner is cordially invited to call the undersigned at (301) 952-1011.

Respectfully submitted,

Date: March 10, 2008
Reg. No. 40,000
Tel. No. (301) 952-1011

D'vorah Graeser, PhD
Agent for Applicant
c/o Discovery Dispatch
9003 Florin Way
Upper Marlboro, Maryland 20772

Attachments

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Reduction in Pulmonary Fibrosis *In Vivo* by Halofuginone

ARNON NAGLER, NATALIE FIRMAN, REGINA FEFERMAN, SHAMAY COTEV, MARK PINES, and SHMUEL SHOSHAN

Departments of Bone Marrow Transplantation, and Anesthesiology, Hadassah University Hospital; Connective Tissue Research Laboratory, Department of Oral Biology, Faculty of Dental Medicine, Hebrew University of Jerusalem; and Institute of Animal Science, Agricultural Research Organization, the Volcani Center, Bet Dagan, Israel

Pulmonary fibrosis is a disorder causing a high mortality rate for which therapeutic options are limited. Therefore, the effect of halofuginone, a novel inhibitor of collagen type I synthesis, on bleomycin-induced pulmonary fibrosis was studied in rats. Pulmonary fibrosis was induced by intraperitoneal injections of bleomycin for seven consecutive days, and halofuginone was administered intraperitoneally every second day during the entire experimental period of 42 d. Collagen determination in the lungs and the examination of histologic sections showed that halofuginone significantly reduced fibrosis relative to the untreated control rats. We conclude that halofuginone is a potent *in vivo* inhibitor of bleomycin-induced pulmonary fibrosis, and that it may potentially be used as a novel therapeutic agent for the treatment of this dysfunction. **Nagler A, Firman N, Feferman R, Cotev S, Pines M, Shoshan S. Reduction in pulmonary fibrosis *in vivo* by halofuginone.** AM J RESPIR CRIT CARE MED 1996;154:1082–6.

Pulmonary fibrosis is a chronic and incurable clinical condition that is a most serious complication in patients following bone marrow transplantation, radiotherapy, and chemotherapy (1–4). Therapeutic options for pulmonary fibrosis are rather few, not specific, and usually of limited success (1, 2, 5–7).

The pathogenesis of pulmonary fibrosis is not completely understood. Several animal models of induced pulmonary fibrosis have been developed including intratracheal or parenteral administration of bleomycin, a drug which is also used as an adjuvant chemotherapeutic agent (4, 7, 8). The lung disease induced by bleomycin is characterized by inflammation of the lower respiratory tract, marked interstitial edema, and alveolar capillary damage which brings about intra-alveolar infiltration of macrophages, mast cells, and inflammatory cells. This may enhance fibroblast proliferation and bring about increased interstitial collagen type I deposition and consequent secondary diffuse thickening of the alveolar septa and fibrosis (4, 9, 10). In certain cases of idiopathic pulmonary fibrosis, as well as in certain animal models, cell-mediated-immunity-to-collagen type I has been reported (11).

Recently, it has been demonstrated that halofuginone, an alkaloid originally isolated from the plant *Dichroa febrifuga* and commonly used as a coccidiostat in chickens and turkeys, suppressed avian skin collagen synthesis *in vivo* (12). Halofuginone also prevented fibrosis in mice that were experimentally induced with graft-versus-host disease (GVHD) and in tight skin mice (TSK) (13), and reduced anastomotic intima hyper asia in rab- bits (14). In culture, halofuginone attenuated expr sion of the procollagen α1(I) gene and brought about a decrea in collagen production by murine and avian skin fibroblasts w out affecting cell proliferation or collagenase activity (15).

This study describes reduction in pulmonary fil osis following systemic administration of halofuginone to bleo ycin-treated rats.

METHODS

Animals and Treatment Protocol

All animal experiments were performed following appr al by the Institutional Committee for Care and Use of Laboratory nimals of the Hebrew University of Jerusalem, and according to the g idelines of the National Institutes of Health on the Care and Use of Labo tory Animals.

Eighty male Sabra rats weighing 350–400 g were use n this experiment. They were divided into groups of 21, as follow

1. Intact control group;
2. Bleomycin (Lundbeck, Denmark)-treated group wh received intraperitoneal injections of 5 mg/kg body weight for ven consecutive days.
3. Halofuginone (Hoechst-Roussel, Germany)-treated pup which received intraperitoneal injections 5 mg/rat every othe lay during the entire experimental period.
4. Bleomycin plus halofuginone-treated group which rec ed both agents in separate syringes (as in 2 and 3) following an i rval of 2 h.

All animals were fed a regular diet and received drinki water *ad libitum*. The weight of the animals and their food intake re monitored during the entire experimental period. From each group animals were killed with an overdose of pentobarbital after 4 and vk. The lungs were dissected and washed free of blood with phosph e-buffered saline. One lung was immediately frozen in liquid nitro n and lyophilized. The dry tissue was then weighed and processed r biochemical analysis. The other lung was put into Carnoy's fixati for histologic and immunohistochemical analysis.

Biochemical Analysis

To assess the effect of halofuginone on bleomycin-inc ed lung fibro-

(*Received in original form November 1, 1995 and in revised form February 5, 1996*)

Supported in part by Israeli Government Ministry of Health Research Grants 514042-1 (A.N.) and 325009 (S.S.).

Correspondence and requests for reprints should be addressed to A. Nagler, M.D., Hadassah University Hospital, Ein Kerem, P.O. Box 12000, Jerusalem 91120, Israel.

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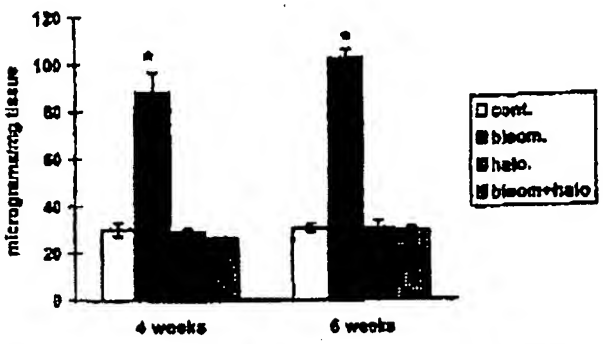

Figure 1. Effect of halofuginone on hydroxyproline concentrations in lungs of bleomycin-treated rats after 4 and 6 wk. *$p < 0.05$ relative to intact control rats.

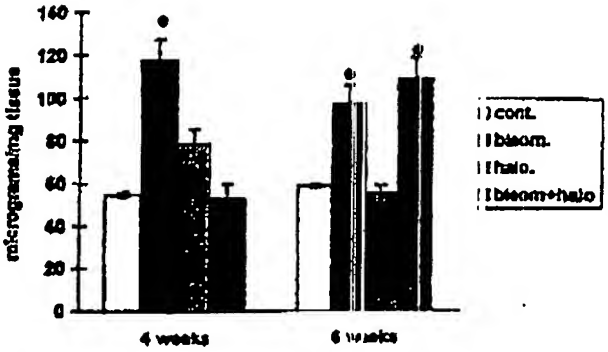

Figure 2. Effect of halofuginone on protein concent tions in lungs of bleomycin-treated rats after 4 and 6 wk. *$p < 0.($ relative to intact control rats.

sis, the collagen and protein contents of the lungs were determined, as follows: the entire dried right lower lobe was weighed and finely minced and homogenized in distilled water and subsequently boiled in distilled water for 30 min. After cooling and centrifugation, the tissue residue was subjected to a second cycle of the extraction, boiling, and centrifugation procedure. Both supernatants thus obtained were pooled, and aliquots were taken for the determination of hydroxyproline (Hypro), as a marker for collagen, and protein by the method of Bradford (16). For Hypro determination, sample aliquots were subjected to acid hydrolysis with 6 N HCl at 138° C for 3 h followed by the assay according to Stegemann and Stalder (17). The results were expressed as micrograms per mg tissue dry weight, and as the Hypro/protein ratio calculated from data obtained from the same tissue aliquot. Quantity of DNA was determined by the method of Burton (18). The statistical significance of the results was determined by the two-sample Student's *t* test for differences in means (19).

Histologic Analysis

The Carnoy-fixed lungs were paraffin-embedded, and histologic sections 6 mm thick were cut from the apex, central part, and lower end of the left lung, and stained with hematoxylin-eosin or Van Gieson's stain for interstitial collagen. Some sections were examined for the presence of mast cell chymase using a monoclonal antibody to rat mast cell chymase, kindly provided by Dr. David Woolley, Manchester, UK. The method employed for the localization of chymase-positive cells was that described by Tetlow and Woolley (20).

RESULTS

The administration of bleomycin for seven consecutive days brought about a significant increase in collagen as measured by hydroxyproline concentrations and protein in the lungs (expressed as μg/mg dry weight) after 4 and 6 wk compared with control (Figures 1 and 2). The hydroxyproline/protein ratio increased significantly from 0.5 + 0.008 in the controls to 0.7 + 0.05 after 4 wk, and 0.9 + 0.075 after 6 wk in the lungs of bleomycin-treated animals (Figure 3).

The administration of halofuginone alone had no effect on hydroxyproline concentration or the Hypro/protein ratio, compared with control animals after 4 and 6 wk (Figures 1–3). However, in the lungs of the bleomycin-treated rats, halofuginone treatment brought about a significant reduction ($p < 0.001$) in the hydroxyproline levels which decreased to that of the controls after 4 and 6 wk (Figure 1). The protein levels were significantly lower in the lungs of bleomycin-treated animals that received halofuginone after 4 wk ($p < 0.003$), but not after 6 wk, compared with bleomycin treatment alone (Figure 2). It is noteworthy that halofuginone had no anorectic effect when administered alone or following bleomycin treatment.

A significant decrease in DNA levels was o erved in the fibrotic lungs of bleomycin-treated rats after 4 w . 6 wk, which was abolished by halofuginone (Figure 4).

Examination of the histologic sections reveale diffuse interstitial pneumonitis and fibrosis in the lungs of : bleomycin-treated animals after 6 wk (Figure 5). However, the lungs of bleomycin-treated animals that also received ha fuginone, an almost normal histologic picture was apparent th very little inflammation or fibrosis (Figure 6).

A surprising finding, although not quantitate in this study, was that of conspicuous decrease in the numb of chymase-positive mast cells in the lungs of bleomycin-tre d animals 4 and 6 wk after bleomycin treatment (Figure 6), mpared with those of the untreated animals. Halofuginone s med to have no effect on the number and distribution of mast lls (Figure 7).

DISCUSSION

Pulmonary fibrosis leads to progressive accumu tion of interstitial connective tissue of which collagen is the major component (21). Tissue repair in animals of higher phyl including humans is preceded by the formation of a fibrous s r, rather than by regeneration. This is no more than a second l st solution to the problem created by interrupting tissue integr , because the fibrosis that replaces complex tissue during hea ng may bring

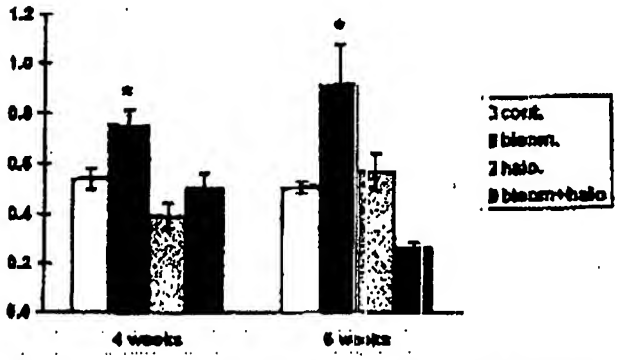

Figure 3. Effect of halofuginone on hyd oxyproline rotein ratio in lungs of bleomycin-treated rats. The ratio was calcu ted from data obtained from the same sample aliquot. *$p < 0.05$ ative to intact control rats.

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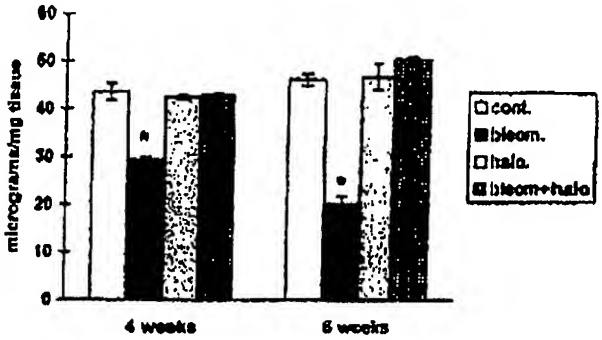

Figure 4. Effect of halofuginone on DNA levels in lungs of bleomycin-treated rats after 4 and 6 wk. *$p < 0.05$ relative to intact control rats.

about functional aberrations (22). Thus, control of excessive fibrosis formation and its related complications is of paramount importance for the health and welfare of the patient. It is well established that collagen synthesis occurs at a higher rate in wounds that in normal tissue immediately following injury (22). This early appearance of newly synthesized collagen in an injured area is of major importance, because the neocollagen is turned over, and the ratio between its synthesis and degradation plays a crucial role in the ultimate outcome of the healing process. The development of therapeutic strategies for lung fibrosis by specifically targeting various aspects of fibrogenesis and collagen type I synthesis may, therefore, be of therapeutic importance.

We used the bleomycin model to induce pulmonary fibrosis, and we showed that the lung architecture could be restored and the interstitial pneumonitis and fibrosis were almost completely abrogated when the animals were administered halofuginone. This was accompanied by a significant reduction in lung collagen content and correlated with the histologic findings indicating an almost complete absence of fibrosis in the lungs of the halofuginone-treated animals. These results are in a reement with our previous observations demonstrating a spec ic inhibition in the expression of collagen type I by halofuginor both *in vitro* (15) and *in vivo* (12, 13). It is of interest to note at the effect of halofuginone on collagen type I was apparen t sites of increased metabolic activities, such as during devel ing fibroses. This is similar to an earlier published observatio on the effect of a beta-receptor blocking agent on increased co gen production during development of a granuloma but nc in the intact skin (23).

The conspicuous decrease in DNA observed i the lungs of bleomycin-treated animals was in agreement with r ults of previous *in vitro* studies in which a decrease in DNA as found in cultured fibroblasts incubated in the presence o arying concentrations of bleomycin (24–26). The increase i DNA which we found following halofuginone administratio night be associated with both the abolition of the bleomycin duced inhibition and the inhibiting effect of halofuginone the fibrotic process which leads to decreased cellularity.

We have no obvious explanation for the obser d, although not quantitated, conspicuous decrease in pulmon y mast cells following bleomycin treatment, as demonstrate by the anti-chymase reaction in all the examined sections from bleomycin-treated animal lungs. It is, however, consistent with rlier reports in which it has been suggested that mast cells we not playing a critical role in initiating or promoting lung fibr is (27), and that bleomycin-induced lung fibrosis is unaffected the absence of mast cells (28, 29). As to the effect of halofu none on the number of mast cells, our observation is consiste with earlier published results indicating no effect of halofuginor on the number of mast cells *in vivo* in murine graft-versus-ho disease (13). In any event, this is currently subject to further vestigation.

Current therapeutic options for pulmonary fibro are limited. Steroids, alkylating agents, nonsteroidal anti-inflam atory preparations, antioxidants, immunosuppressive drugs, nicillamine, and colchicine have been used for treatment of pulm ary fibrosis with little success (11, 30, 31). Antiproteases and ll adhesion molecules have been tried as well, but were nonspec c, and some of them were found to be toxic (32). As to the th apeutic role

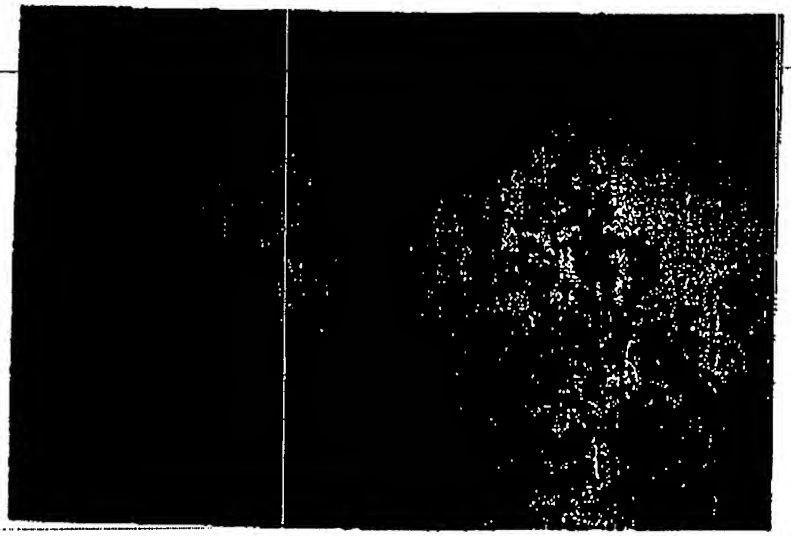

Figure 5. Photomicrograph showing a section of lung from a bleomycin-treated control rat after 6 wk. Note diffuse pneumonitis, thickened alveolar walls (*arrows*), and abundant interstitial collagen (*arrowheads*). Hematoxylin-eosin stain. Original magnification: ×125.

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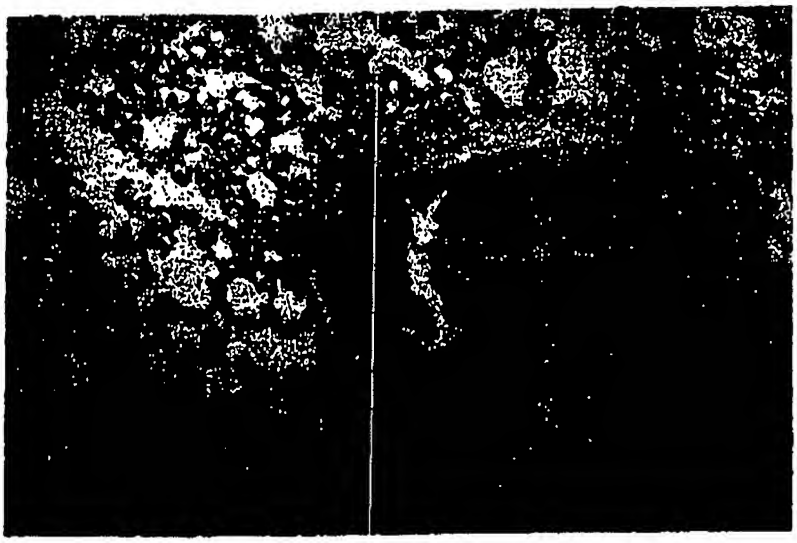

Figure 6. Photomicrograph showing a section of lung from bleomycin-treated control rat. The section was processed for immunohistochemical detection of mast cells using antichymase antibodies. Note conspicuous sparsity of mast cells after 6 wk (*arrow*). Alkaline phosphatase and hematoxylin staining. Original magnification: ×125.

of the antioxidants, tissue injury by oxidants and proteases may not be key factors leading to pulmonary fibrosis (5).

Recently, researchers have been partially successful in using the bleomycin-induced pulmonary fibrosis model for the evaluation of the antifibrotic effect of anti–tissue necrosis factor (anti-TNF), anti-interleukin-1 receptors (anti-IL-1R), anti–transforming growth factor β (anti-TGFβ) antibodies, as well as anti-interferon γ (anti-IFNγ) (33–35).

Inhibitors of collagen synthesis, deposition, and cross-linking have been tried as antifibrosis factors in the past (11). However, this approach of nonspecifically inhibiting collagen production may result in major adverse effects. In contrast, halofuginone, at very low doses is a specific inhibitor of collagen type I (12–15), and presumably effective during increased metabolic activity, which means that it might be harmless to tissue with lower turnover rates. This, obviously, deserves further investigation.

Based on the present experimental results, we propose to consider halofuginone as a potential novel therapeutic agent for re-

Figure 7. Photomicrograph showing a section of lung from a bleomycin-treated rat who received halofuginone. The section was processed for immunohistochemical detection of mast cells as described in METHODS. Note normally appearing airspaces (AS) as well as pronounced antichymase-positive reaction indicating presence of mast cells after 6 wk (*arrows*). Alkaline phosphatase and hematoxylin staining. Original magnification: ×125.

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vention and probably also for treatment of pulmonary fibrosis in a variety of clinical situations. Because it is unlikely that a single drug will suffice for adequate treatment of the whole spectrum of pulmonary fibrosis, one has to look for additives and possibly synergistic effects between halofuginone and other molecules, such as anticytokines, anti-TGFβ in particular, and cytokine receptor antibodies in the hope of finding the best treatment for lung fibrosis and other fibrotic conditions.

References

1. Krowka, M. J., E. C. Rosenow, and H. C. Hoagland. 1985. Pulmonary complications of bone marrow transplantation. *Chest* 87:237–246.
2. Breuer, R., I. S. Lossos, and G. E. Epler. 1992. Bone marrow transplantation related pulmonary complications. *Pulmon. Crit. Care Update* 7:3–8.
3. Seiden, M. V., A. Elias, L. Ayash, M. Hunt, J. P. Eder, L. E. Schnipper, E. Freei, III, and K. H. Antman. 1992. Pulmonary toxicity associated with high dose chemotherapy in the treatment of solid tumors with autologous marrow transplant: an analysis of four chemotherapy regimens. *Bone Marrow Transplant.* 10:57–63.
4. Adamson, I. Y. R., and D. H. Bowden. 1974. The pathogenesis of bleomycin-induced pulmonary fibrosis in mice. *Am. J. Pathol.* 77: 185–198.
5. Phan, S. H. 1995. New strategies for treatment of pulmonary fibrosis. *Thorax* 50:415–421.
6. Hunninghake, G. W., and A. R. Kalica. 1995. Approaches to the treatment of pulmonary fibrosis. *Am. J. Respir. Crit. Care Med.* 151: 915–918.
7. Snider, G. L., B. R. Celli, R. H. Goldstein, J. J. O'Brien, and E. C. Lucey. 1978. Chronic interstitial pulmonary fibrosis produced in hamsters by endotracheal bleomycin. *Am. Rev. Respir. Dis.* 117:289–297.
8. Doherty, D. E., N. Hirose, L. Zagarella, and R. M. Cherniack. 1992. Prolonged monocyte accumulation in the lung during bleomycin-induced pulmonary fibrosis: a noninvasive assessment of monocyte kinetics by scintigraphy. *Lab Invest.* 66:231–242.
9. Hay, J. S., S. Shahzeidi, and G. Laurent. 1991. Mechanisms of bleomycin-induced lung damage. *Arch. Toxicol.* 65:81–94.
10. Goto, T., D. Befus, R. Low, and J. Bienenstock. 1984. Mast cell heterogeneity and hyperplasia in bleomycin-induced pulmonary fibrosis in rats. *Am. Rev. Respir. Dis.* 130:797–802.
11. Phan, S. H. 1989. Diffuse interstitial fibrosis. *In* D. Massaro, editor. Lung Cell Biology. Marcel Dekker, New York. 907–979.
12. Granot, I., I. Bartov, I. Plavnik, E. Wax, S. Hurwitz, and M. Pines. 1991. Increased skin tearing in broilers and reduced collagen synthesis in skin *in vivo* and *in vitro* in response to the coccidiostat halofuginone. *Poult. Sci.* 70:1559–1563.
13. Levi-Schaffer, F., A. Nagler, S. Slavin, V. Knopov, and M. Pines. 1995. Inhibition of collagen synthesis and changes in skin morphology in murine graft-versus-host disease and tight skin mice: effect of halofuginone. *J. Invest. Dermatol.* 106:84–88.
14. Choi, E. T., A. D. Callow, N. L. Sehgal, D. M. Brown, and U. S. Ryan. 1995. Halofuginone, a specific collagen type I inhibitor, reduces anastomotic intima hyperplasia. *Arch. Surg.* 130:257–261.
15. Granot, I., S. Hurwitz, O. Halevy, and M. Pines. 1993. Halofuginone: an inhibitor of collagen type I synthesis. *Biochim. Biophys. Acta* 1156:107–112.
16. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* 72:248–254.
17. Stegemann, H., and K. Stalder. 1967. Determination of hydroxyproline. *Clin. Chim. Acta* 18:267–273.
18. Burton, K. 1968. Determination of DNA concentration with diphenylamine. *Meth. Enzymol.* XII-B:163–166.
19. Marani, A. 1972. Methods of Planning and Conduction of Experiments. Academon Press, Jerusalem. 33.
20. Tetlow, L. C., and D. E. Woolley. 1995. Distribution, activation and tryptase/chymase phenotype of mast cells in the rheumatoid lesion. *Ann. Rheum. Dis.* 54:549–555.
21. Shoshan, S., and J. Gross. 1974. The biosynthesis and metabolism of collagen and its role in tissue repair processes. *Isr. J. Med. Sci.* 10:537–568.
22. Shoshan, S. 1981. Wound healing. *Int. Rev. Conn. Tissue Res.* 9:1–26.
23. Shoshan S., B. Saks, A. Shifter, and S. Pisanti. 1990. The effect of β-receptor blocking agent, Cyclosporin A, and calcium antagonist (Nifedipin) on collagen production in granulation tissue *in vivo*. *Ann. N.Y. Acad. Sci.* 580:570–572.
24. Umezawa, H., M. Ishizuka, K. Maeda, and T. Takeuchi. 1967. Studies on bleomycin. *Cancer* 20:891–895.
25. Otsuka, K., S. Murota, and Y. Mori. 1976. Stimulatory effect of bleomycin on the synthesis of acidic glycosaminoglycans in cultured fibroblasts derived from rat carrageenin granuloma. *Biochim. Biophys. Acta* 444:359–368.
26. Clark, J. G., B. C. Starcher, and J. Ulito. 1980. Bleomycin-induced synthesis of type I procollagen by human lung and skin fibroblasts in culture. *Biochim. Biophys. Acta* 631:359–370.
27. Zhang, K., M. Gharaee-Kermani, M. L. Jones, J. S. Warren, and S. H. Phan. 1994. Lung monocyte chemoattractant protein-1 gene expression in bleomycin-induced pulmonary fibrosis. *J. Immunol.* 153:4733–4741.
28. Mori, H., K. Kawada, P. Zhang, Y. Uesugi, O. Sakamoto, and Koda, A. 1991. Bleomycin-induced pulmonary fibrosis in genetically mast cell-deficient $WBB6F_1$-W/W^v mice and mechanism of the suppressive effect of tranilast, an antiallergic drug inhibiting mediator release from mast cell, on fibrosis. *Int. Arch. Allergy Appl. Immunol.* 95:195–201.
29. Botana, L. M., E. Arnaez, M. R. Vieytes, A. Alfonso, and M. C. Louzao. 1992. Nonimmunological release of histamine from rat mast cells elicited by antineoplastic agents: effect of drug combinations. *Cancer Chemother. Pharmacol.* 30:487–490.
30. Cross, C. E., D. Warren, J. E. Gerrets, D. W. Wilson, B. Halliwul and J. A. Last. 1985. Deferroxamine injection does not affect bleomycin-induced lung fibrosis in rats. *J. Lab. Clin. Med.* 106:433–438
31. Sendelbach, L. E., R. C. Lindenschmidt, and H. P. Witschi. 1985. The effect of Cyclosporin A on pulmonary fibrosis induced by butylated hydroxytoluene, bleomycin and beryllium sulfate. *Toxicol. Lett.* 26 163–173.
32. Nagai, A., K. Aoshiba, Y. Ishihara, H. Inano, K. Sakamoto, and E Yamaguchi. 1992. Administration of α1-proteinase inhibitor ameliorates bleomycin-induced pulmonary fibrosis in hamsters. *Am. Rev Respir. Dis.* 145:651–656.
33. Piguet, P. F., M. A. Collart, G. E. Grau, Y. Kapanci, and P. Vassali 1989. Tumor necrosis factor/cachectin plays a key role in bleomycin induced pneumopathy and fibrosis. *J. Exp. Med.* 170:655–663.
34. Raghow, R., P. Irish, and A. H. Kang. 1989. Coordinate regulation of transforming growth factor β gene expression and cell proliferation in hamster lungs undergoing bleomycin-induced pulmonary fibrosis. *J. Clin. Invest.* 84:1836–1842.
35. Giri, S. N., and D. M. Hyde. 1988. Ameliorating effect of an interferon inducer polyinosinic-polycytidylic acid on bleomycin-induced lung fibrosis in hamsters. Morphologic and biochemical evidence. *Am J. Pathol.* 133:525–536.

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Inhibition of fibroblast to myofibroblast transition by halofuginone contributes to the chemotherapy-mediated antitumoral effect

Yuval Sheffer,[1] Oded Leon,[1] Jehonathan H. Pinthus,[2] Arnon Nagler,[3] Yoram Mor,[4] Olga Genin,[1] Maya Iluz,[1] Norifumi Kawada,[5] Katsutoshi Yoshizato,[6] and Mark Pines[1]

[1]Institute of Animal Science, Agricultural Research Organization, Volcani Center, Bet Dagan, Israel; [2]Department of Surgery, McMaster University, Hamilton, Ontario, Canada; [3]Division of Hematology and [4]Department of Urology, Chaim Sheba Medical Center, Tel Hashomer, Israel; [5]Department of Hepatology, Graduate School of Medicine, Osaka City University, Osaka, Japan; and [6]Developmental Biology Laboratory, CLUSTER Project, and 21st Century COE Program, Department of Biological Science, Graduate School of Science, Hiroshima University, Higashi-Hiroshima, Japan

Abstract

Stromal myofibroblasts play an important role in tumor progression. The transition of fibroblasts to myofibroblasts is characterized by expression of smooth muscle genes and profuse synthesis of extracellular matrix proteins. We evaluated the efficacy of targeting fibroblast-to-myofibroblast transition with halofuginone on tumor progression in prostate cancer and Wilms' tumor xenografts. In both xenografts, low doses of halofuginone treatment, independent of the route of administration, resulted in a trend toward inhibition in tumor development. Moreover, halofuginone synergizes with low dose of docetaxel in prostate cancer and vincristine and dactinomycin in Wilms' tumor xenografts, resulting in significant reduction in tumor volume and weight comparable to the effect observed by high doses of the respective chemotherapies. In prostate cancer and Wilms' tumor xenografts, halofuginone, but not the respective chemotherapies, inhibited the synthesis of collagen type I, α-smooth muscle actin, transgelin, and cytoglobin, all of which are characteristics of activated myofibroblasts. Halofuginone, as the respective chemotherapies, increased the synthesis of Wilms' tumor suppressor gene product (WT-1) and prostate apoptosis response gene-4 (*Par-4*), resulting in apoptosis/necrosis. These results suggest that targeting the fibroblast-to-myofibroblast transition with halofuginone may synergize with low doses of chemotherapy in achieving a significant antitumoral effect, avoiding the need of high-dose chemotherapy and its toxicity without impairing treatment efficacy. [Mol Cancer Ther 2007;6(2):570–7]

Introduction

Most solid tumors consist of a mixture of neoplastic and nonneoplastic cells together with extracellular matrix (ECM) components. This cellular microenvironment directly modulates tissue architecture, cell morphology, and cell fate (1, 2), and the ECM-stromal cell interaction contributes to the neoplastic phenotype (3). The conversion of fibroblasts into myofibroblasts, as mediated by transforming growth factor-β1 (TGF-β1), is the most prominent stromal reaction in a large number of epithelial lesions (4–6). In addition to the major increase in ECM components, the fibroblasts that acquire an activated phenotype, the myofibroblasts are characterized by expression of smooth muscle genes such as α-smooth muscle actin (*αSMA*) and *transgelin* (*SMA22α*; refs. 4, 7). The myofibroblasts are associated with the tumor cells at all stages of cancer progression (8) and, in various malignancies, tumor-dependent differentiation of fibroblasts toward myofibroblasts further promotes neoplastic progression (9–12).

It is well established that collagen type I, the major ECM component produced by myofibroblasts, not only functions as a scaffold for the tissue but also regulates the expression of genes associated with cellular signaling and metabolism, gene transcription, and translation, thus affecting fundamental cellular processes that are essential for tumor progression (13). Collagen type I accumulation has been observed at the tumor-stroma boundary (2, 14), and the TGF-β–dependent activation of neighboring stroma cells leads to a survival advantage and increased metastasis formation (15). Collagen type I has been hypothesized to be a signal for invasion, and its intratumoral expression level has been associated with increased tumor invasiveness (16, 17). The association of collagen type I with stellate cell activation–associated protein, also known as cytoglobin (Cygb/STAP), which has been hypothesized to regulate collagen synthesis under the control of TGF-β, is characteristic of cells that exhibit an activated phenotype (18, 19).

Cancer patients often face aggressive chemotherapy that involves multiple-treatment regimens, which are associated with significant side effects that adversely affect

Received 8/7/06; revised 11/26/06; accepted 12/12/06.

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Note: Y. Sheffer and O. Leon contributed equally to this work.
This article is a contribution from the Agricultural Research Organization, Volcani Center, Bet Dagan, Israel.

Requests for reprints: Mark Pines, Institute of Animal Sciences, Volcani Center, P.O. Box 6, Bet Dagan, 50250, Israel. Phone: 972-8-9484408; Fax: 972-8-9475075. E-mail: pines@agri.huji.ac.il

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doi:10.1158/1535-7163.MCT-06-0468

the patient's quality of life. Thus, an ideal anticancer treatment should simultaneously target both the malignant cells and their microenvironment to inhibit tumor growth, development, and invasion. Until recently, the lack of specific inhibitor(s) of any component of the ECM, in general, and of collagen type I, in particular, limited the success of such an approach. Halofuginone, an analogue of the plant alkaloid febrifugine, is an inhibitor of collagen type I synthesis (for review, see refs. 20, 21 and references therein). Halofuginone blocks TGF-β–mediated collagen synthesis by decreasing activation of Smad3 through increased expression of Smad7, an inhibitor of Smad2/3 activation, and by a c-jun–dependent mechanism (22–24). In culture, halofuginone attenuated collagen synthesis by skin fibroblasts from scleroderma and chronic graft-versus-host disease (cGvHD) patients. In animal models of fibrosis (adhesions, cGvHD, radiation-induced fibrosis, and pulmonary fibrosis), administration of halofuginone prevented the increase in collagen α1(I) gene expression and collagen synthesis. In the liver, halofuginone inhibited the synthesis of collagen type I and αSMA, resulting in inhibition of stellate cell activation and liver fibrosis (25). Human clinical efficacy was shown by topical dermal administration of halofuginone in patients with scleroderma and cGvHD (21). Halofuginone-dependent inhibition of collagen type I synthesis was associated with inhibition of microvessel formation *in vitro* and *in vivo* and resulted in decreased tumor growth in multiple murine models (26), including transplantable and chemically induced bladder carcinoma, glioma, von-Hippel-Lindau–associated pheochromocytoma (27), prostate cancer (28), and Wilms' tumor (29).

In the present study, we targeted the fibroblast-to-myofibroblast transition with halofuginone and evaluated its antitumoral effect alone or in combination with low dose of chemotherapy in human prostate and Wilms' tumor xenografts.

Materials and Methods

Materials

FCS, DMEM, and trypsin-EDTA solution (0.02–0.25%) were obtained from Biochemical Industries (Bet Haemek, Israel). Sirius red F3B was obtained from BDH Laboratory Supplies (Poole, United Kingdom). Halofuginone bromhydrate was obtained from Collgard Biopharmaceuticals Ltd. (Tel Aviv, Israel). Polyclonal rabbit anti-human prostate apoptosis response gene-4 (Par-4) and anti–Wilms' tumor suppressor gene product (WT-1) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Proliferating cell nuclear antigen staining kit was obtained from Zymed Laboratories (San Francisco, CA). Monoclonal antibodies to αSMA were from DAKO A/S (Glostrup, Denmark). Antibodies to Cygb/STAP were prepared as previously described (18). Dactinomycin (Cosmegen) was from Merck & Co., Inc. (West Point, PA); vincristine was from Pharmachemie BV (Haarlem, the Netherlands), and docetaxel (Taxotere) was from Aventis Pharma (Surrey, United Kingdom).

Animals and Experimental Design

All animal experiments were carried out according to the guidelines of the Volcani Center Institutional Committee for Care and Use of Laboratory Animals. Nude (CD1 *nu/nu*) male mice (Harlan Laboratories, Jerusalem, Israel) were housed in cages (four per cage) under conditions of constant photoperiod (12 light:12 dark) with free access to food and water. During the experiments, tumor size was determined with a caliper (formula: length × width × depth × 0.5236) and is presented as mean ± SE. Prostate cancer xenografts were established by implanting the human cell line PC3 (American Type Culture Collection, Rockville, MD) in Matrigel (4×10^6 cells/mL) s.c. using a 27-gauge needle (28). Treatments were initiated when the tumors reached a volume of 100 mm^3. Halofuginone was given in the diet at a concentration of 3 ppm throughout the experiment (~0.15–0.30 mg/kg). Docetaxel in saline (6 or 12.5 mg/kg) was administered i.v. into the tail vein on day 0 and 15 days later, and the control groups were treated with saline alone. The Wilms' tumor xenografts were established from tumors derived from a 3-year-old female with a stage I favorable histology tumor, in accordance with the National Wilms' Tumor Study Group. The establishment and maintenance of the Wilms' tumor xenografts were previously described in detail (29). Wilms' tumor cells (3×10^6) in Matrigel were injected s.c. and treatment was initiated 4 days later. Halofuginone (at 2 μg/mouse) was injected i.p. every other day. Chemotherapy was given as low doses of dactinomycin (0.525 mg/mL saline) once a week and of vincristine (0.583 mg/mL saline) once every 3 weeks, or as high doses of dactinomycin (1.575 mg/mL saline) once a week and of vincristine (1.75 mg/mL saline) once every 3 weeks.

Preparation of Sections, *In situ* Hybridization, and Immunohistochemistry

At the end of the experiments, tumors were collected and fixed overnight in 4% paraformaldehyde in PBS at 4°C. Serial 5-μm sections were prepared and embedded in Paraplast. Samples were stained with Sirius red for collagen. *In situ* hybridization for collagen α1(I) was done as previously described (25). The primers used for preparing the transgelin (SMA22α) probe were F-GGCCAACA-AGGGTCCATCCTAT and R-AGGACATTGGCTTCCAA-GGACA.

The sense probes elicited no signal. Immunohistochemistry was done with anti–Par-4 (1:500), anti–WT-1 (1:1,000), anti–Cygb/STAP (1:500), and anti-αSMA (1:200) antibodies. Peroxidase activity was revealed by using 3,3′-diaminobenzidine as chromogen.

Cell Culture and Northern Blots

The androgen-independent human prostate PC3 cancer cells were cultured in DMEM with 10% FCS in the presence of various concentrations of halofuginone. Total RNA was extracted with TRIzol reagent after 6 and 12 h, and *Par-4* gene expression was analyzed by Northern blotting. The primers used were F-GCAGATCGAGAA-GAGGAAGC and R-CATAAAAGGTGGCACACATCA, which resulted in a 998-bp probe.

Table 1. Effect of halofuginone, alone and in combination with docetaxel (Taxotere), on tumor development in prostate cancer xenografts

Treatment	Day 10	Day 21	Day 26	Day 30
	Volume $(mm)^3$			Weight (g)
Control	310 ± 60^a	450 ± 90^a	582 ± 111^a	1.56 ± 0.3^a
Halofuginone	126 ± 48^a	201 ± 97^{ab}	254 ± 95^{ab}	0.70 ± 0.2^{ab}
Taxotere (6 mg/kg)	247 ± 74^a	331 ± 97^{ab}	327 ± 89^{ab}	0.81 ± 0.2^{ab}
Halofuginone + Taxotere (6 mg/kg)	155 ± 51^a	173 ± 55^b	182 ± 63^b	0.46 ± 15^b
Taxotere (12.5 mg/kg)	226 ± 75^a	185 ± 50^b	186 ± 48^b	0.47 ± 10^b

NOTE: During the experiments, tumor volume was determined with caliper and tumor weight was evaluated at the end of the experiment. The results are presented as mean ± SE of five to seven animals in each group. In each column, means without a common superscript differ significantly ($P < 0.05$) according to Duncan's multiple range test.

Statistical Analysis and Image Analysis

Data were subjected to one-way ANOVA and means were separated by Duncan's multiple range test. Fibrosis levels, as exhibited by Sirius red staining, were analyzed by ImagePro software (Media Cybernetics, Inc. Silver Spring, MD). At least five sections taken from different animals were used and the mean results were expressed as arbitrary units.

Results

Effects of Combination Therapy on Tumor Development

Two animal models were used to evaluate the effect of inhibition of fibroblast activation by halofuginone, alone or in combination with low doses of chemotherapy, on tumor progression. In the control animals with prostate cancer xenograft, tumors derived from PC3 cells reached a mean volume of 582 mm^3 after 26 days and a mean weight of 1.56 g after 30 days. A trend in the reduction of tumor volume and weight was achieved with low doses of either halofuginone (3 ppm in the diet) or docetaxel (6 mg/kg) alone (Table 1). When the two treatments were combined, both the tumor volume and weight were significantly lower than those in the controls and reached the levels of tumors treated with a high dose of docetaxel (12.5 mg/kg). The same tendency was observed in the Wilms' tumor xenograft model: the control tumors reached a mean volume of 686 mm^3 84 days after tumor cell implantation (Table 2), and a low dose of halofuginone or of chemotherapy (dactinomycin and vincristine) caused a reduction in tumor volume at any point of evaluation. When the treatments were combined, a significant reduction in tumor volume as compared with that in the controls was observed, and it reached comparable measurements as in tumors treated with a high dose of chemotherapy. These results suggest that the combination of systemic treatment with a low dose of halofuginone, either given in the diet or injected i.p. together with a low dose of chemotherapy, results in the same therapeutic effect as high-dose chemotherapy alone.

Effect of Halofuginone on Fibroblast Activation

Acquisition of an activated phenotype by fibroblasts is associated with increased ECM production, especially of collagen type I and its regulator Cygb/STAP, and increased expression of the smooth muscle genes *αSMA* and *transgelin*. In both xenografts, the control, untreated tumors exhibited all the characteristics of activated fibroblasts [i.e., a large number of cells expressing the collagen (α1) type I gene resulting in high levels of collagen deposition (Fig. 1), as well as a large number of cells that synthesize Cygb/STAP and αSMA (Fig. 2)]. In addition,

Table 2. Effect of halofuginone, alone and in combination with chemotherapy, on tumor development in Wilms' tumor xenografts

Treatment	Day 66	Day 72	Day 75	Day 84
	Volume (mm^3)			
Control	71 ± 32^a	330 ± 50^a	338 ± 107^a	686 ± 100^a
Halofuginone	44 ± 31^a	80 ± 50^{ab}	84 ± 50^{ab}	125 ± 20^{ab}
Chemotherapy (low)	26 ± 12^a	56 ± 29^{ab}	67 ± 20^{ab}	99 ± 20^{ab}
Halofuginone + chemotherapy (low)	17 ± 3.0^a	24 ± 8.4^b	24 ± 8.5^b	30 ± 11^b
Chemotherapy (high)	11 ± 1.7^a	16 ± 2.3^b	18 ± 2.7^b	17 ± 2.6^b

NOTE: Chemotherapy was given at low doses of dactinomycin (0.525 mg/mL) once a week and vincristine (0.583 mg/mL) once every 3 wks or at high doses of dactinomycin (1.575 mg/mL) once a week and vincristine (1.75 mg/mL) once every 3 wks. During the experiments, tumor volume was determined with a caliper. The results are presented as mean ± SE of five to seven animals in each group. In each column, means without a common superscript differ significantly ($P < 0.05$) according to Duncan's multiple range test.

the activated fibroblasts in the Wilms' tumor xenografts expressed high levels of the *transgelin* (*SMA22α*) gene (Fig. 2). No transgelin expression was observed in the prostate cancer tumors of naïve or treated mice (data not shown). According to its unique mode of action, halofuginone inhibited the synthesis of all of these genes, both in the prostate cancer and in the Wilms' tumor xenografts, which suggests a similar mechanism of action. For example, collagen content (Sirius red staining) was inhibited with halofuginone alone by 2.3- and 2.0-fold and with αSMA by 2.5- and 1.7-fold in prostate cancer and Wilms' tumor xenografts, respectively. On the other hand, the expression of these genes and the synthesis of their proteins were not affected by the respective chemotherapies (docetaxel in prostate cancer, dactinomycin plus vincristine in Wilms' tumor xenografts). However, when the mice were treated with halofuginone and the respective chemotherapy, almost complete inhibition of synthesis of collagen (5.0- and 3.1-fold in prostate cancer and Wilms' tumor xenografts, respectively) and αSMA Cygb/STAP (4.1- and 3.5-fold in prostate cancer and Wilms' tumor xenografts, respectively) and of *transgelin* gene expression was observed (Table 3; Figs. 1 and 2).

Effect of Combination Therapy on WT-1 and Par-4

The *TGF-β1* gene is under the control of the Wilms' tumor suppressor gene product (WT-1) via the WT-1/Egr-1 consensus element in its promoter (30). The level of WT-1 was low in the untreated Wilms' tumor and prostate cancer xenografts (Fig. 3), but both halofuginone and the respective chemotherapy treatments caused major increases in WT-1 levels. In the prostate cancer tumors, an additive effect on WT-1 levels was observed when the mice were treated with both halofuginone and docetaxel whereas no such effect was observed in Wilms' tumor xenografts. However, in both xenografts, halofuginone and the respective chemotherapy caused reductions in tumor cell proliferation, which resulted in apoptosis/necrosis, as indicated by proliferating cell nuclear antigen staining. When the treatments were given together, only a small number of the tumor cells still maintained their cell cycle (Fig. 4). WT-1, through its zinc-finger DNA binding domain, interacts with the prostate apoptosis response gene-4 (Par-4) leucine repeat domain (31). Both halofuginone and docetaxel, alone and in combination, increased the level of Par-4 in PC3 xenografts by 9.2-, 9.5-, and 10.1-fold, respectively (Table 3; Fig. 4). In addition, halofuginone increased *Par-4* gene expression in PC3 cells in culture in a dose-dependent manner at all time points tested (Fig. 4). Par-4 was not detected in the Wilms' tumor xenografts before or after treatment either with halofuginone or with dactinomycin and vincristine, or with the two treatments in combination (data not shown).

Discussion

Increasing survival rates of cancer patients and the trend toward higher doses of toxic multimodality therapies have stimulated a focusing on the health-related quality of life of

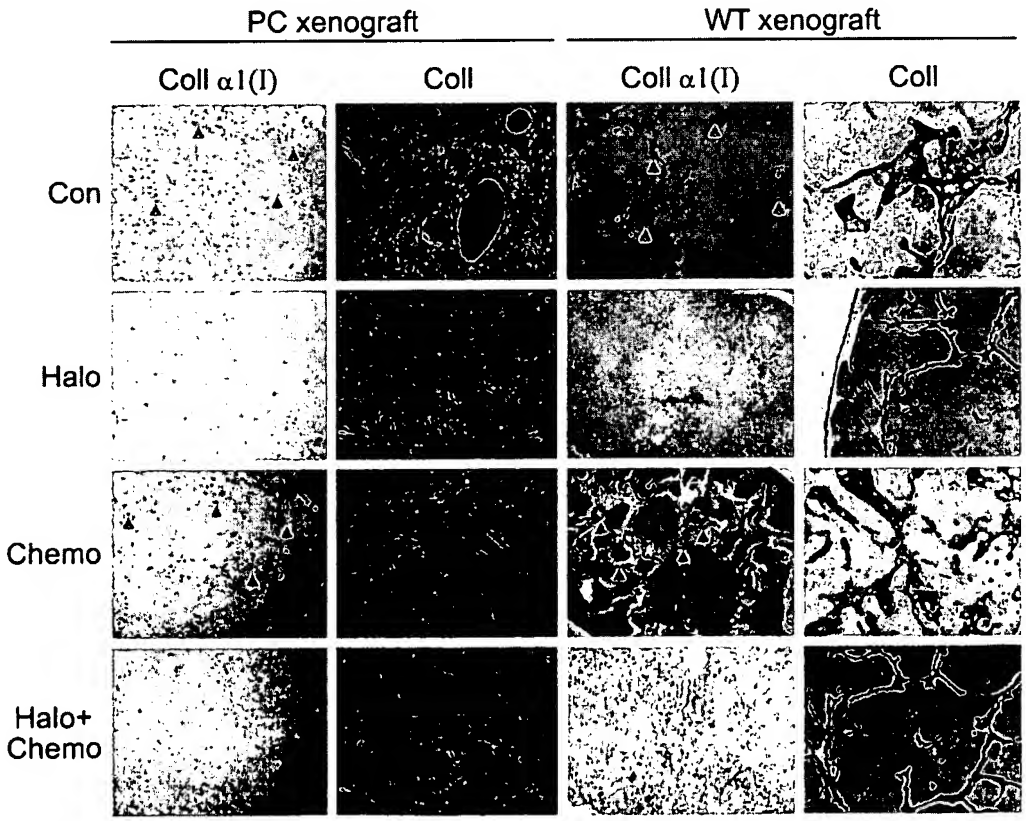

Figure 1. Collagen type I gene expression and collagen content in prostate cancer and Wilms' tumor xenografts. The untreated mice (*Con*) expressed a high level of collagen α1(I) gene (*arrows*) and a high level of collagen, as evaluated by *in situ* hybridization and Sirius red staining (collagen stained red), respectively. Halofuginone (*Halo*), alone or in combination with chemotherapy (*Halo + Chemo*), but not the respective chemotherapies alone (*Chemo*), attenuated collagen α1(I) gene expression and collagen content.

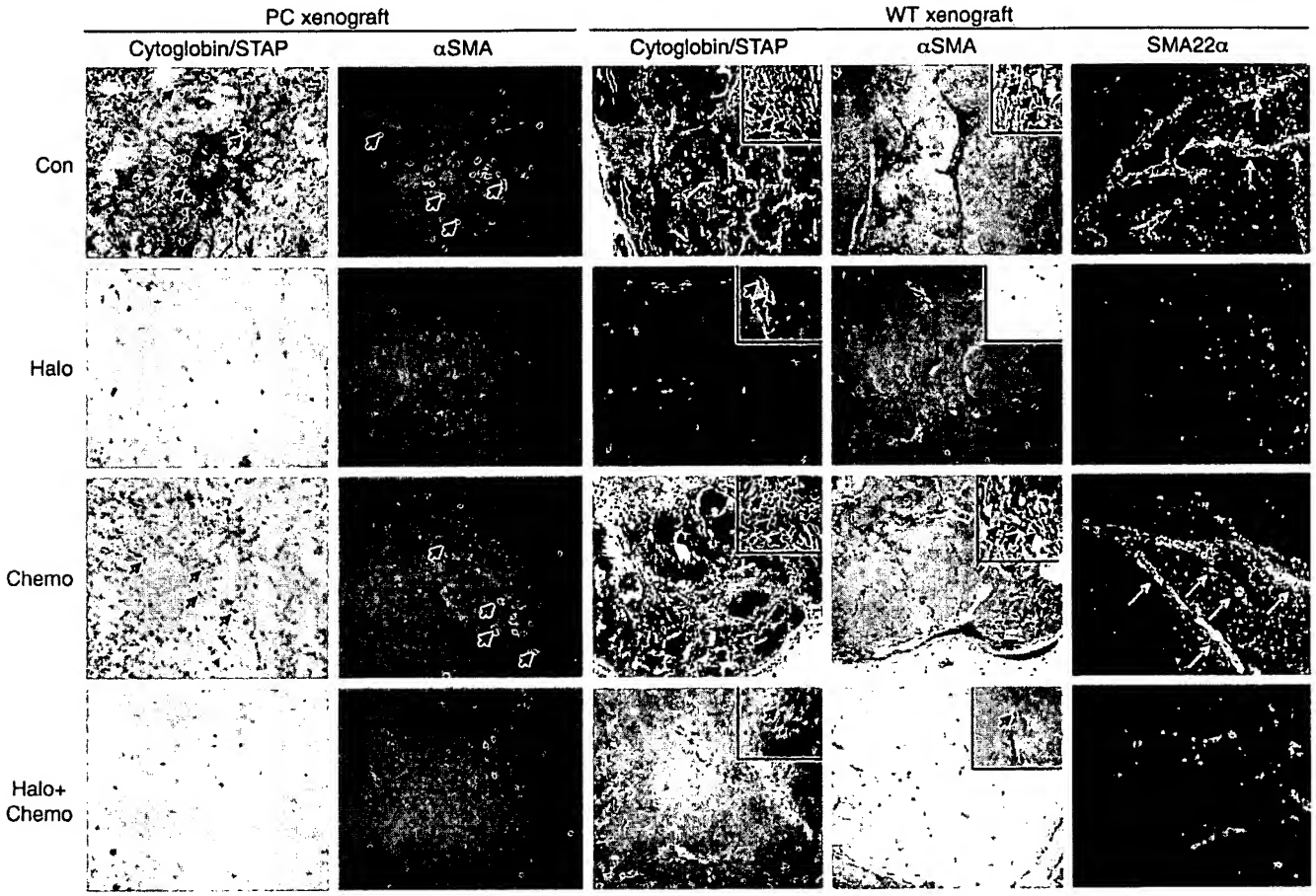

Figure 2. Cygb/STAP, αSMA, and transgelin in prostate cancer and Wilms' tumor xenografts. The untreated mice exhibit high levels of Cygb/STAP and αSMA (*arrows*), as evaluated by immunohistochemistry. Halofuginone, alone or in combination with chemotherapy, but not the respective chemotherapies alone, attenuated Cygb/STAP and αSMA levels. The untreated Wilms' tumor xenograft expressed high levels of the transgelin gene, as evaluated by *in situ* hybridization, which were inhibited by halofuginone, but not by dactinomycin and vincristine.

cancer survivors, which may continue to be of concern long after treatment has been discontinued. This applies especially in relation to Wilms' tumor pediatric patients, for whom the aim of most recent clinical trials has been to reduce the overall treatment burden, as the achievement of long-term survival by means of the heavy treatment burden is frequently accompanied by severe early and late complications (32). Myofibroblasts or cancer-associated fibroblasts together with ECM components provide the microenvironment that is pivotal for cancer cell growth, tumor invasion, and metastatic progression (8). Thus, inhibition of fibroblast activation may become a viable approach for tumor treatment, especially when combined with chemotherapy. In the prostate cancer and Wilms' tumor xenografts, low doses of halofuginone, independent of the route of administration, together with low doses of the respective chemotherapies, were as efficacious as the high chemotherapy doses (Tables 1 and 2). Thus, targeting the ECM and its cell population with halofuginone enables reduction in the chemotherapy dose without impairing the efficacy of the treatment. This is probably because diversity of modes of action is a prerequisite for a successful combination therapy. In prostate cancer and Wilms' tumor xenografts, halofuginone, but not the respective chemotherapies, affected collagen α1(I) gene expression and collagen content. These results are consistent with our previous findings that halofuginone inhibited collagen type I synthesis in various preclinical studies in which excess of collagen was the hallmark of the disease (20, 21), and halofuginone-dependent inhibition of collagen type I synthesis was correlated with reduction in tumor progression (26, 27). In addition, only halofuginone inhibited the synthesis of Cygb/STAP, which is known to regulate collagen synthesis under the control of TGF-β (18, 19), a finding that is consistent with the notion that halofuginone acts through the inhibition of Smad2/3 phosphorylation downstream of TGF-β (22–24). The expression of Cygb/STAP was up-regulated in fibrotic lesions of the pancreas and kidney in which activated fibroblast-like cells or myofibroblasts are known to proliferate (18). In the liver, Cygb/STAP is synthesized in activated stellate cells that are αSMA positive (33). Cygb/STAP probably is involved in

Table 3. Effect of halofuginone, alone and in combination with chemotherapy, on collagen, αSMA, Cygb/STAP, and Par-4 in prostate cancer and Wilms' tumor xenografts

Protein	Treatment			
	Control	Halofuginone	Chemotherapy	Halofuginone + chemotherapy
Collagen (arbitrary units)				
PC	0.85 ± 0.05[a]	0.37 ± 0.04[b]	0.80 ± 0.06[a]	0.17 ± 0.04[b]
WT	0.70 ± 0.05[a]	0.35 ± 0.05[b]	0.72 ± 0.04[a]	0.23 ± 0.03[b]
αSMA (arbitrary units)				
PC	0.61 ± 0.04[a]	0.24 ± 0.02[b]	0.65 ± 0.05[a]	0.15 ± 0.01[b]
WT	0.54 ± 0.08[a]	0.32 ± 0.04[b]	0.51 ± 0.04[a]	0.15 ± 0.03[b]
Cygb/STAP (arbitrary units)				
PC	0.92 ± 0.11[a]	0.45 ± 0.04[b]	0.89 ± 0.07[a]	0.23 ± 0.01[b]
WT	0.74 ± 0.11[a]	0.33 ± 0.08[b]	0.71 ± 0.08[a]	0.21 ± 0.01[b]
Par-4 (arbitrary units)				
PC	0.03 ± 0.01[a]	0.27 ± 0.06[b]	0.29 ± 0.04[b]	0.30 ± 0.04[b]

NOTE: Image analysis using ImagePro software was done on at least five sections taken from different animals and the mean results were expressed as arbitrary units. In each line, means without a common superscript differ significantly ($P < 0.05$) from the control according to Duncan's multiple range test. Abbreviations: PC, prostate cancer; WT, Wilms' tumor.

cellular oxygen homeostasis and supply and plays a role as an O_2 reservoir that is used under hypoxic conditions, such as those occurring in fibrotic tissues and tumors with insufficient oxygen supply. Cygb/STAP expression is up-regulated under hypoxia and is regulated by the hypoxia-inducible factor 1 (19), which regulates angiogenesis and is overexpressed in human cancers and their metastases (34). The inhibition of angiogenesis by halofuginone, in general (26), and of prostate cancer and Wilms' tumor xenografts, in particular (28, 29), may suggest the involvement, at least in part, of hypoxia-inducible factor 1–dependent Cygb/STAP overexpression in angiogenesis and tumor progression.

The fibroblast-myofibroblast transdifferentiation is associated with the expression of αSMA and transgelin (SMA22α). In hepatic fibrosis, halofuginone inhibited the activation of liver stellate cells, which is characterized by

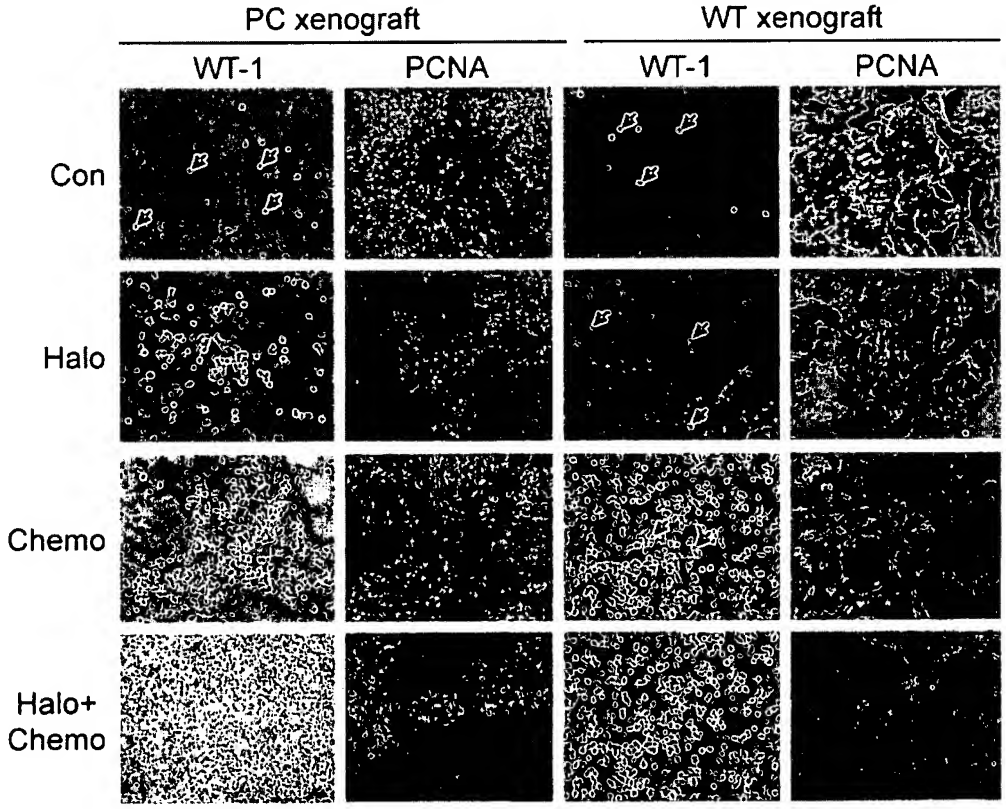

Figure 3. WT-1 and tumor cell cycle. A low level of WT-1 was observed in the untreated mice, as shown by immunohistochemistry (*arrows*). Both halofuginone and the respective chemotherapy caused increases in the level of WT-1. Most of the cells in the untreated mice are in their proliferative state, as indicated by immunohistochemistry with anti–proliferating cell nuclear antigen (*PCNA*) antibodies. Halofuginone and chemotherapy treatments caused apoptosis/necrosis, and when the mice were treated with both halofuginone and chemotherapy, almost no proliferative cells were observed.

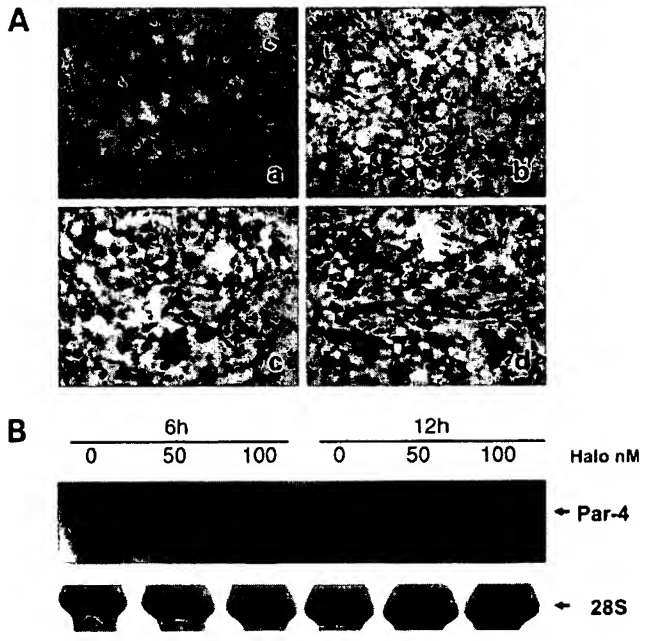

Figure 4. Par-4 in prostate cancer xenograft. **A**, immunohistochemistry of Par-4 in control mice (**a**), mice treated with halofuginone (**b**), mice treated with docetaxel (**c**), and mice treated with halofuginone in combination with docetaxel (**d**). **B**, PC3 cells were incubated with halofuginone and, at the end of the experiment, *Par-4* gene expression was detected by Northern blot analysis. The 28S rRNA was used as a control of RNA loading.

their transformation from quiescent, vitamin A–storing cells to highly proliferative, αSMA-positive cells that synthesize large amounts of collagen type I (25). The high levels of *transgelin* gene expression in the Wilms' tumor xenografts and the high levels of αSMA-positive fibroblasts in prostate cancer and Wilms' tumor control, untreated xenografts were inhibited by halofuginone, but not by the respective chemotherapies (Fig. 2). It is important to note that the αSMA-positive activated fibroblasts in the prostate cancer tumors did not express transgelin, and that even in the Wilms' tumor tumors, not all the αSMA-positive cells expressed transgelin, which suggests not only that the stromal fibroblasts differ from myofibroblasts of the reactive stroma but also that the myofibroblasts themselves are not a homogeneous population. This should be taken into account in strategic planning to inhibit the fibroblast-to-myofibroblast transition as a novel modality for cancer therapy. As transgelin and αSMA are regulated by TGF-β via the Smad3 pathway (35–37), halofuginone, an inhibitor of Smad3 phosphorylation, is probably an excellent candidate for such a strategy.

Previously, we showed that halofuginone stimulated the *in vitro* synthesis of the zinc-finger suppressor gene *WT-1* by Wilms' tumor and prostate cancer cells. In addition, halofuginone-dependent inhibition of Wilms' tumor growth was associated with increased synthesis of WT-1 (29). Overexpression of the *WT-1* gene has been observed in various tumors, although its specific role and those of its alternative splicing variants in tumorigenesis are not clear (38–40). In PC3 cells, halofuginone increased the level of the WT-1 54-kDa product but lowered that of the truncated 36-kDa product (data not shown). WT-1-induced apoptosis has been implicated in the actual development of the tumor (38, 41). In prostate cancer, WT-1 suppressed tumor cell growth, regulated the androgen-signaling pathway, and inhibited vascular endothelial growth factor expression, all of which are essential for tumor growth inhibition (42, 43). Although halofuginone induced a significant increase in WT-1 synthesis in both xenografts used, the respective chemotherapies induced WT-1 synthesis to a greater extent (Fig. 3). The halofuginone-dependent increase in WT-1 synthesis may be due to the interrelationships between WT-1 and TGF-β, which function via the WT-1/Egr-1 consensus element of the human TGF-β (30). The increases in WT-1 synthesis elicited by halofuginone and the respective chemotherapies were associated with a decrease in the number of proliferating cells and increased apoptosis/necrosis of tumor cells, especially when the treatments were applied in combination (Fig. 3). Thus, the beneficial effect of the combination therapy is probably greater than can be deduced from tumor size alone (Tables 1 and 2). The association of WT-1 and apoptosis in the prostate cancer xenograft was mediated, at least in part, by Par-4, one of the WT-1–interacting proteins that is functionally required, but is not sufficient, for apoptosis (31, 44). Halofuginone and the respective chemotherapies increased Par-4 in the prostate cancer xenograft and halofuginone increased *Par-4* gene expression in the PC3 cell line (Fig. 4).

At present, halofuginone is being evaluated in cancer clinical trials. Systemically, therapeutically effective plasma levels can be reached at a dosage that is well tolerated. No dose-limited toxicities were observed at 1 mg/d and the recommended dose for phase II studies of halofuginone is 0.5 mg administered daily (45). In cancer patients, the absorption of halofuginone after oral drug administration was associated with maximum peak drug levels at 3.4 ± 4.8 h. The estimated terminal elimination half-life had mean values of 28.3 ± 12.9 h, and only 4% to 12% of the administered dose of halofuginone was excreted unchanged in the urine, mainly in the first 24 h after drug administration. The area under the curve reached at the recommended dose of 0.5 mg/d is within the range in which antitumor efficacy was observed in preclinical studies (45). Consequently, thanks to the unique mode of action of halofuginone, its use in combination therapy, in association with other cancer-combating drugs that exhibit different modalities, is expected to successfully lower the dosages of chemotherapeutic drugs or to use less toxic compounds, thus offering reductions in the treatment burden imposed on cancer patients.

References

1. van Kempen LC, Rhee JS, Dehne K, Lee J, Edwards DR, Coussens LM. Epithelial carcinogenesis: dynamic interplay between neoplastic cells and their microenvironment. Differentiation 2002;70:610–23.

2. van Kempen LC, Ruiter DJ, van Muijen GN, Coussens LM. The tumor microenvironment: a critical determinant of neoplastic evolution. Eur J Cell Biol 2003;82:539 – 48.

3. Shekhar MPV, Pauley R, Heppner G. Host microenvironment in breast cancer development: extracellular matrix-stromal cell contribution to neoplastic phenotype of epithelial cells in the breast. Breast Cancer Res 2003;5:130 – 5.

4. Ronnov-Jessen L, Villadsen R, Edwards JC, Petersen OW. Differential expression of a chloride intracellular channel gene, CLIC4, in transforming growth factor-β1-mediated conversion of fibroblasts to myofibroblasts. Am J Pathol 2002;161:471 – 80.

5. Lewis MP, Lygoe KA, Nystrom ML, et al. Tumour-derived TGF-β1 modulates myofibroblast differentiation and promotes HGF/SF-dependent invasion of squamous carcinoma cells. Br J Cancer 2004;90:822 – 32.

6. Micke P, Ostman A. 2004 Tumour-stroma interaction: cancer-associated fibroblasts as novel targets in anti-cancer therapy? Lung Cancer 2004;45 Suppl 2:S163 – 75.

7. Untergasser G, Gander R, Lilg C, Lepperdinger G, Plas E, Berger P. Profiling molecular targets of TGF-β1 in prostate fibroblast-to-myofibroblast transdifferentiation. Mech Ageing Dev 2005;126:59 – 69.

8. Kalluri R, Zeisberg M. Fibroblasts in cancer. Nat Rev Cancer 2006;6: 392 – 401.

9. DeClerck YA. 2000 Interactions between tumour cells and stromal cells and proteolytic modification of the extracellular matrix by metalloproteinases in cancer. Eur J Cancer 2000;36:1258 – 68.

10. De Wever O, Mareel M. Role of myofibroblasts at the invasion front. Biol Chem 2002;383:55 – 67.

11. Tuxhorn JA, Ayala GE, Smith MJ, Smith VC, Dang TD, Rowley DR. Reactive stroma in human prostate cancer: induction of myofibroblast phenotype and extracellular matrix remodeling. Clin Cancer Res 2002;8: 2912 – 23.

12. Zidar N, Gale N, Kambic V, Fischinger J. Proliferation of myofibroblasts in the stroma of epithelial hyperplastic lesions and squamous carcinoma of the larynx. Oncology 2002;62:381 – 5.

13. Kiefer J, Alexander A, Farach-Carson MC. Type I collagen-mediated changes in gene expression and function of prostate cancer cells. Cancer Treat Res 2004;118:101 – 24.

14. van Kempen LC, Rijntjes J, Claes A, et al. Type I collagen synthesis parallels the conversion of keratinocytic intraepidermal neoplasia to cutaneous squamous cell carcinoma. J Pathol 2004;204:333 – 9.

15. Berking C, Takemoto R, Schaider H, et al. Transforming growth factor-β1 increases survival of human melanoma through stroma remodeling. Cancer Res 2001;61:8306 – 16.

16. van Hoorde L, van Aken E, Mareel M. Collagen type I: a substrate and a signal for invasion. Prog Mol Subcell Biol 2000;25:105 – 34.

17. Lockwood DS, Yeadon TM, Clouston AD, et al. Tumor progression in hepatocellular carcinoma: relationship with tumor stroma and parenchymal disease. J Gastroenterol Hepatol 2003;18:666 – 72.

18. Nakatani K, Okuyama H, Shimahara Y, et al. Cytoglobin/STAP, its unique localization in splanchnic fibroblast-like cells and function in organ fibrogenesis. Lab Invest 2004;84:91 – 101.

19. Schmidt M, Gerlach F, Avivi A, et al. Cytoglobin is a respiratory protein in connective tissue and neurons, which is up-regulated by hypoxia. J Biol Chem 2004;279:8063 – 9.

20. Pines M, Vlodavsky I, Nagler A. Halofuginone - a novel anti-fibrotic therapy. Gen Pharmacol 1997;30:445 – 50.

21. Pines M, Snyder D, Yarkoni S, Nagler A. Halofuginone to treat fibrosis in chronic graft versus host disease and scleroderma. Biol Blood Marrow Transplant 2003;9:417 – 25.

22. McGaha TL, Kodera T, Spiera H, Stan AC, Pines M, Bona CA. Halofuginone inhibition of COL1A2 promoter activity via a c-Jun-dependent mechanism. Arthritis Rheum 2002;46:2748 – 61.

23. McGaha TL, Phelps RG, Spiera H, Bona C. Halofuginone, an inhibitor of type-I collagen synthesis and skin sclerosis, blocks transforming-growth-factor-β-mediated Smad3 activation in fibroblasts. J Invest Dermatol 2002;118:461 – 70.

24. Xavier S, Piek E, Fujii M, et al. Amelioration of radiation-induced fibrosis: inhibition of transforming growth factor-β signaling by halofuginone. J Biol Chem 2004;279:15167 – 76.

25. Bruck R, Genina O, Aeed H, Alexiev R, Nagler A, Pines M. Halofuginone to prevent and treat thioacetamide-induced liver fibrosis in rats. Hepatology 2001;3:379 – 86.

26. Elkin M, Miao HQ, Nagler A, et al. Halofuginone: a potent inhibitor of critical steps in angiogenesis progression. FASEB J 2000;14:2477 – 85.

27. Pines M, Vlodavsky I, Nagler A. Halofuginone: from veterinary use to human therapy. Drug Dev Res 2000;50:371 – 8.

28. Gavish Z, Pinthus JH, Barak V, et al. Growth inhibition of prostate cancer xenografts by halofuginone. Prostate 2002;51:73 – 83.

29. Pinthus JH, Sheffer Y, Nagler A, Mor Y, Genina O, Pines M. Inhibition of Wilm's tumor xenografts progression by halofuginone is accompanied by activation of WT1 gene expression. J Urol 2005;174: 1527 – 31.

30. Dey BR, Sukhatmem VP, Roberts AB, et al. Repression of the transforming growth factor-β1 gene by the Wilms' tumor suppressor WT1 gene product. Mol Endocrinol 1994;8:595 – 602.

31. Sells SF, Han SS, Muthukkumar S, et al. 1997 Expression and function of the leucine zipper protein Par-4 in apoptosis. Mol Cell Biol 1997;17:3823 – 32.

32. Kalapurakal JA, Dome JS, Perlman EJ, et al. Management of Wilms' tumour: current practice and future goals. Lancet Oncol 2004;5:37 – 46.

33. Kawada N, Kristensen DB, Asahina K, et al. Characterization of a stellate cell activation-associated protein (STAP) with peroxidase activity found in rat hepatic stellate cells. J Biol Chem 2001;276: 25318 – 23.

34. Hirota K, Semenza GL. Regulation of angiogenesis by hypoxia-inducible factor 1. Crit Rev Oncol Hematol 2006;59:15 – 26.

35. Ronnov-Jessen L, Petersen OW. Induction of α-smooth muscle actin by transforming growth factor-β1 in quiescent human breast gland fibroblasts. Implications for myofibroblast generation in breast neoplasia. Lab Invest 1993;68:696 – 707.

36. Hu B, Wu Z, Phan SH. Smad3 mediates transforming growth factor-β-induced α-smooth muscle actin expression. Am J Respir Cell Mol Biol 2003;29:397 – 404.

37. Qiu P, Ritchie RP, Fu Z, et al. Myocardin enhances Smad3-mediated transforming growth factor-β1 signaling in a CArG box-independent manner: Smad-binding element is an important cis element for SM22α transcription *in vivo*. Circ Res 2005;97:983 – 91.

38. Lee SB, Haber DA. Wilms tumor and the WT1 gene. Exp Cell Res 2001;264:74 – 99.

39. Loeb DM, Sukumar S. The role of WT1 in oncogenesis: tumor suppressor or oncogene. Int J Hematol 2002;76:117 – 26.

40. Tuna M, Chavez-Reyes A, Tari AM. HER2/neu increases the expression of Wilms' Tumor 1 (WT1) protein to stimulate S-phase proliferation and inhibit apoptosis in breast cancer cells. Oncogene 2005;24:1648 – 52.

41. Morrison DJ, English MA, Licht JD. WT1 induces apoptosis through transcriptional regulation of the proapoptotic Bcl-2 family member Bak. Cancer Res 2005;65:8174 – 82.

42. Graham K, Li W, Williams BR, Fraizer G. Vascular endothelial growth factor (VEGF) is suppressed in WT1-transfected LNCaP cells. Gene Expr 2006;13:1 – 14.

43. Fraizer G, Leahy R, Priyadarshini S, Graham K, Delacerda J, Diaz M. 2004 Suppression of prostate tumor cell growth *in vivo* by WT1, the Wilms' tumor suppressor gene. Int J Oncol 2004;24:461 – 71.

44. Richard DJ, Schumacher V, Royer-Pokora B, Roberts SG. 2001 Par-4 is a coactivator for a splice isoform-specific transcriptional activation domain in WT1. Genes Dev 2001;15:328 – 39.

45. de Jonge MJA, Dumez H, Verweij J, Yarkoni S, et al. Phase I and pharmacokinetic study of halofuginone, an oral quinazolinone derivative in patients with advanced solid tumours. Eur J Cancer 2006;42: 1768 – 74.